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Timing is everything – Choosing the right time to screen the herd for neosporosis

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ABSTRACT

Neosporosis is a major cause of abortions in cattle, leading to economic losses. As no effective treatment or vaccine is currently available, the only strategy to reduce the impact of neosporosis in endemic herds is implementation of control programs, based on serological surveillance. This study aimed to determine the optimal timing for serological testing. Sera were collected from 132 heifers at three farms at three months intervals, between the ages 5 and 28 months. *Neospora* serological status and antibody titers were evaluated by IFAT, and by ELISA on the first sampling. The agreement between ELISA and IFAT results was 89.9 % (Kappa=0.798). Overall *Neospora* seroprevalence ranged between 36 % and 66 % on different dates. The antibody titers of almost all heifers fluctuated over time. Of 91 heifers that were sampled on 5 occasions, 67 % maintained their serological status throughout the study. Most cases of negative heifers that become positive in one or more tests (19 of 21 heifers) were transient, while only three remained persistently infected in all further tests. Seropositivity was significantly lower in lactating cows than in pregnant cows or heifers. The results of the first sampling date were representative of the overall status on all dates, with an agreement of 94.5 % (Kappa=0.889). The results demonstrate how fluctuations in antibody titers may affect the sensitivity of serological surveillance. Sampling young heifers, prior to their first insemination may be advisable, allowing future discrimination between what appear to be new infection, which is mostly transient, and congenital or chronic infections, which are usually life-long. This information may assist in the implementation of effective control measures in the herd.

1. Introduction

Neospora caninum is a unicellular eukaryotic parasite which is a cyst-forming coccidia and a major cause of abortions in cattle (Dijkstra et al., 2003; Dubey et al., 2017; Dubey, 2003; Dubey and Schares, 2011). Canids are the definitive hosts of *N. caninum* and parasite oocysts are secreted in their feces. Cattle and several other animal species may act as intermediate hosts. Infection occurs as a result of ingestion of food or water contaminated with oocysts. Parasitemia in the intermediate hosts is usually short, and may result in clearance or in long-term carriage of parasites within tissue cysts (Dubey et al., 2017; Dubey, 2003; Dubey and Schares, 2011). Infected cows do not show clinical signs of disease. However, immunosuppression during pregnancy may evoke re-emergence of dormant parasites and infection the placenta and fetus (Innes et al., 2001). Infection of the fetus during pregnancy may lead to fetal death and abortion or to the birth of congenitally infected calve (Dubey et al., 2017; Innes et al., 2001; Mazuz et al., 2011). This route of vertical transmission is extremely efficient in cattle, and considered to be the main source of infection in endemic herds (Dubey and Schares, 2011; Pare et al., 1996; Sanchez-Sanchez et al., 2021; Schares et al., 1998).

Chronically infected cows have increased risk of abortions and repeated abortions. Moreover, the risk of abortion correlates with the cow's anti-*Neospora* antibody titer (Mazuz et al., 2014, 2015). Previous studies demonstrated that dams with antibody titers of 1:800 or higher had increased risk of abortions, while dams with lower antibody titers did not (Mazuz et al., 2021). Antibody titers of chronically infected cows fluctuate over time, and may be influenced by the animal's immune status (Almeria et al., 2017; Eastick and Elsheikha, 2010; Innes et al., 2001; Pare et al., 1997; Takashima et al., 2013). These fluctuations influence the sensitivity of diagnostic tests, especially in cows with lower titers.

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Neosporosis is prevalent in most parts of the world and causes considerable economic losses due to abortions and repeated abortions (Larson et al., 2004; Nayeri et al., 2022; Reichel et al., 2013). The high rates of vertical transmission perpetuate the problem in endemic herds, as considerable rates of replacement heifers are borne congenitally infected (Davison et al., 1999; More et al., 2009; Schares et al., 1998). Currently, no effective treatment or vaccine is available for neosporosis. The only strategy that could be implemented to reduce the impact of neosporosis in the herd is implementation of prevention of and control measures (Dubey et al., 2007; Reichel et al., 2020). Such control programs usually include increased biosecurity to reduce the risk of horizonal transmission and either removal from the herd or selective breeding of infected cows, in order to reduce the risk of vertical transmission (Dubey et al., 2007). Regardless of its details, any control program is based on initial serological screening or the herd, in order to detect infected cows.

Serological testing for neosporosis is the best way to detect infected cows, since parasitemia is short, and parasites cannot be detected in blood when they are harbored within tissue cysts. Detection of antibodies is possible in milk or blood, and several methods are available, including several commercial kits. Immunofluorescence antibody test (IFAT) is a widely used quantitative method to detect antibody titer. However, it is relatively labor-intensive, and interpretation of the results requires skill. Enzyme-linked immunosorbent assay (ELISA) is also well-validated, and several commercial kits are available. Although ELISA results may also be quantitative, the commercial kits interpreted them as dichotomous (yes/no) results. Most commercial kits do not state their cutoff titer for seropositivity, and the cutoff titer also varies between scientific studies (Nayeri et al., 2022). Other studies showed varying agreement between kits and methods (Baszler et al., 2001; Campero et al., 2018; Reichel and Pfeiffer, 2002; von Blumroder et al., 2004; Waldner et al., 2004).

The design of serological screening also varies between farms. Some perform point-prevalence survey, where the whole herd is tested at once. Others use a specific time-point in the cow's life for individual testing. Testing replacement heifers may allow detection of positive animals prior their first insemination, which may allow informed decision-making regarding the pregnancy. However, testing during pregnancy may more accurately reflect the risk of abortion during pregnancy. Lastly, serological screening in milk is practically more convenient, but could only be performed after first calving. The combination of fluctuating antibody titers of infected cows and the differences in sensitivity between methods, makes the timing of serological surveillance and the choice of serological method crucial in the interpretation of the results and implementation of control measures.

This study was designed to assess the prevalence of neosporosis and the fluctuations in anti-*Neospora* antibody titers in young heifers up to their first calving, in order to determine the best suited time for serologic surveillance.

2. Methods

2.1. Study design

The study was conducted in three farms with the farm manager's consent and approved by the Animal Experiments Welfare Committee of the Kimron Veterinary Institute (101 2023).

The study was set as a prospective study, which was conducted at three farms known to be highly prevalent to neosporosis. Heifers were serologically tested for neosporosis on three-months intervals between the ages of approximately six months and two years. By the end of the study most heifers were either lactating or at late pregnancy.

2.2. Study population

The study population comprised of 132 Holstein heifers, between 37 and 54 on each farm. Heifers were aged 5 – 9 months during the first sampling. This age was selected to represent each heifer's immune status, after the depletion of maternal antibodies (Hietala and Thurmond, 1999). The same group of heifers were re-sampled 6 times, at 3 months intervals, between June 2022 and January 2024. The number of heifers sampled on each farm is specified in Table 1, along with their ages on each sampling date.

All three farms had a history of neosporosis, which was confirmed by serological testing of a sample of the herd. The farms were selected according to the cooperation of the farm managers and the attending veterinarians. The herd size of farm 1 was approximately 480 cows, while farms 2 and 3 had approximately 330 cows. On farm 2 the young heifers were reared on one location, and were transferred to another after confirmation of pregnancy. All farms used computerized herd management software, and data was collected at each timepoint regarding the exact age, their reproductive status, number of inseminations, day of pregnancy and days in milking (when relevant).

2.3. Sample collection and serological testing

Blood was collected from the tail vein of each heifer into a sterile serum collection tube without anticoagulant. Serum was separated following centrifugation at 4000 g for 4 min, and kept frozen (at −20°C) until testing.

All blood samples were tested for the presence of anti-*Neospora* spp. antibodies by immunofluorescence antibody test (IFAT), as previously described (Shkap et al., 2002). In brief the antigen was prepared in-house using culture-derived NcIs491 tachyzoites (Fish et al., 2007) with an anti-cattle IgG secondary antibody (A8917, Sigma-Aldrich, Darmstadt, Germany), and the results were interpreted using a fluorescent microscope. All sera were tested at 1:200, 1:400, 1:800, 1:3200 and 1:12,800 dilutions with bovine serum albumin (BSA). The endpoint titer was considered as the highest dilution exhibiting fluorescence of the whole *Neospora* organism.

In addition to IFAT, all 132 samples collected at the first timepoint were serologically tested for the presence of anti-*Neospora* antibodies using a commercial competitive enzyme-linked immunosorbent assay (cELISA) kit (ID Screen® *Neospora caninum* Competition, IDvet, Grabels, France), according to the manufacturer's instructions.

2.4. Statistical analysis

The agreement between the serological detection using IFAT and cELISA was evaluated using spearman's correlation (ρ) and Cohen's kappa. Sensitivity, specificity, positive and negative predictive values were calculated for cELISA in reference to IFAT as the gold standard. These calculations were performed using two IFAT cutoff values for positivity, 1:200 and 1:800. The higher cut-off was selected, since it was shown to be the clinically relevant titer, linked to abortions (Mazuz

Table 1
The number of heifers sampled on each sampling date at each of the three farms. The same heifers were sampled on each occasion (if available). The heifers' age range, median and inter quartile range (IQR) are specified for each sampling date.

Sample	1	2	3	4	5	6
Date	Jun-Jul 2022	Oct 2022	Jan 2023	Jun 2023	Sep 2023	Jan 2024
Age range (m)	5–9	10–14	11–16	16–20	20–24	24–28
Median age (IQR)	7 (1.2)	11.2 (1.5)	13.5 (1.4)	18.2 (1.3)	21.4 (1.3)	25.0 (1.6)
Farm 1	37		33	38	34	23
Farm 2	54	53	53	47	48	38
Farm 3	41		38	41	38	30
Total	132	53	124	126	120	100

et al., 2014, 2015, 2021).

The associations between *Neospora* serological status and the farm, sampling date and the cows' reproductive stage were evaluated using Chi square or Fisher's exact tests, as appropriate, and odds ratios (OR) were calculated. The correlation between cow age and serological status as well as the square root of the serological titer were tested using spearman's rho (ρ). The distribution of the square root of the serological titer was compared between sampling dates, farms and reproductive status using the non-parametric Kruskal-Wallis test.

The association between the cows' age and reproductive status and seropositivity were also assessed using generalized estimating equation model (GEE), with a logit link function, where each sample was defined as the subject, and the farm and cow as within subject effects.

The analyses were performed using SPSS 27.0® (IBM Corp, Armonk, NY, USA) and Win Pepi 11.43® statistical software (Abramson, J.H. WINPEPI updated: computer programs for epidemiologists, and their teaching potential. Epidemiologic Perspectives & Innovations, 2011, 8:1). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Serological detection of neosporosis using IFAT and ELISA

The sera collected from the 132 heifers during the first sampling date were tested for the presence of anti-*Neospora* antibodies both by IFAT and cELISA (Table 2).

Forty one of the 132 heifers (31.1 %) were found seronegative via IFAT. The antibody titers of the 68.9 % seropositive heifers ranged between 1:200 and 1:12,800 [median = 800, inter-quartile range (IQR)= 3200]. A total of 73 heifers (55.3 %) had clinically relevant titers of 1:800 or higher (Table 2).

The seroprevalence of neosporosis in the heifers, evaluated by ELISA was 49.2 %. Three samples (2.3 %) were defined as "suspected", and the remaining 48.5 % tested seronegative (Table 2).

When considering 1:200 as the diagnostic titer for seropositivity, the agreement between tests was 78.3 % (Kappa=0.565, $P < 0.001$). When considering IFAT as the "gold standard", the sensitivity and specificity of the ELISA kit were 70.8 % and 95 % respectively, and its positive (PPV) and negative (NPV) predictive values were 96.9 % and 59.4 %, respectively.

The performance of the ELISA kit was considerably better when the cutoff titer was set as 1:800. For this, clinically relevant, cutoff the agreement between tests was 89.9 % (Kappa=0.798, $P < 0.001$), the ELISA sensitivity and specificity were 86.1 % and 94.7 % respectively, and its PPV and NPV were 95.4 % and 84.4 %, respectively.

3.2. Fluctuations of anti-Neospora antibody titers over time

Neospora seropositivity was evaluated using a cutoff titer of 1:800, since the ELISA test used for routine diagnosis is more reliable for this cutoff. In addition, this is the titer associated with abortions. Therefore, titers of 1:200 and 1:400 were considered "borderline".

Neospora overall seroprevalence ranged between 36 % and 66 % on different dates (Fig. 1). Negative samples ranged between 7.9 % and 37 %, while 13.6–29.8 % of the heifers had borderline titers (Fig. 1).

The reproductive status of the heifers gradually changed from "heifer" to "pregnant" and to "lactating" (Fig. 2). By the end of the study period none of the heifer remained barren. Six heifers aborted during the study period, four of them were removed from the herd, on three cases the abortion was the reason. These four heifers were last sampled on days 88–183 of pregnancy (the exact dates of pregnancy lost were not recorded). Four of the six aborting heifers had antibody titers over 1:800 throughout the study (for three these a titer of 1:12,800 was measured on 1–3 occasions).

A total of 91 heifers were sampled on all 5 occasions (excluding the second sampling date, which included only one farm). Forty-seven (51.6 %) of these heifers tested seropositive on the first sampling date (Table 3A). The antibody titers of almost all heifers fluctuated over time. Thirty-nine (83 %) of these heifers tested seropositive on at least four of the five sampling dates. Of these, 21 (45 %) were seropositive on all five sampling dates. In most cases of seroconversion, titers dropped to "borderline", and only four samples (8.5 % of heifers) tested negative on one occasion (Table 3A).

Forty-four (48.4 %) of the heifers tested seronegative on the first sampling date (Table 3B). Forty-one (93.2 %) of these heifers were also seronegative on the last sampling date. Only two (4.5 %) remained seronegative throughout the study, while 22 (50 %) did not cross the 1:800 cutoff for positivity during the study. Most heifers that seroconverted to positive (19 heifers, 43.2 %), reverted to negative by the end of the study period. Only three (6.8 %) remained positive on the last sampling date (Fig. 3B).

3.3. Factors associated with antibody titers

The association between various potential risk factors and *Neospora* seropositivity is summarized in Table 4. The seroprevalence was significantly lower in the last sampling date compared to all other dates ($p < 0.001$), except for the third. The age of the heifers did not correlate with seropositivity (rho=-0.009, $p = 0.817$). The seroprevalence did not differ significantly between other dates. The seroprevalence in lactating cows was lower than in pregnant cows (but not in heifers, $p = 0.005$).

The overall seroprevalence in farm 1 (42.4 %) was lower than in farms 2 (60.4 %) and 3 (58.4 %) ($p < 0.003$). The seroprevalence in farm 1 did not differ significantly between sampling dates ($p = 0.894$), but the fluctuations between dates were significant in farms 2 ($p = 0.008$) and 3 ($p = 0.005$). In both, the prevalence was lowest on the last sampling (Fig. 3).

When the reproductive status and age were analyzed in a multivariable model (GEE), while the cow and farm were included as "within

Table 2
The agreement between the serologic detection of neosporosis by IFAT and ELISA in 132 heifers aged 5–9 months from three farms.

		ELISA Negative	Suspected	Positive	Total
IFAT	**0**	38	1	2	41 (31.1)
	1:200	9	0	0	9 (6.8)
	1:400	7	1	1	9 (6.8)
	1:800	7	0	19	26 (19.7)
	1:3200	3	1	28	32 (24.2)
	1:12800	0	0	15	15 (11.4)
	Total	64 (48.5)	3 (2.3)	65 (49.2)	132 (100)

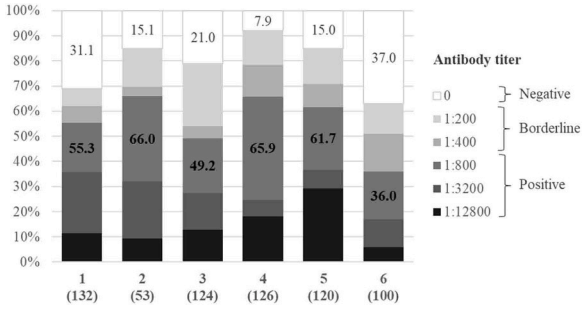

Fig. 1. The seroprevalence of *Neospora caninum* in dairy heifers sampled on six different occasion at three months intervals at three farms. The number of heifer sampled on ech occsion appears in brckets underneath each colomn. Antibody titers were evaluated by IFAT. Titer of 1:800 or higher were considered positive, while titers of 1:200 or a:400 were considered borderline. The seroprevalence (%) on each sampling date appears in bold and the rate (%) of negative samples is specified.

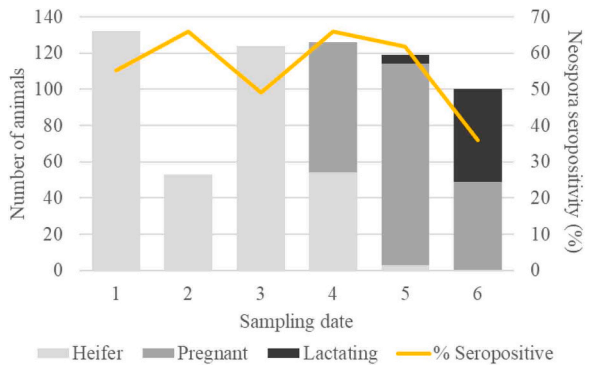

Fig. 2. The reproductive status (columns) of the study population of 132 heifers on each of the six sampling dates, between the ages of 6–9 months and 24–28 months, along with the overall *Neopspora* seropositivity at each sampling date (line).

subject" variables in a multivariable model (GEE), lactation was the only factor found to be significantly associated with seropositivity ($p = 0.006$).

3.4. The use of a single versus repeated screenings

The overall serological status of each of the 91 heifers sampled on five occasions, was set as the majority of samples (three samples with the same status, regardless of the date).

The agreement between the results of the first sampling date and the overall status was 94.5 % (Kappa=0.889). The sensitivity, specificity, PPV and NPV were all above 93 % (Table 5A).

The agreement between the results of the last sampling date and the overall status was 78.0 % (Kappa=0.568). The specificity and NPV were all above 96.5 %, however, the sensitivity and PPV were 60.4 % and 68.9 %, respectively (Table 5B).

4. Discussion

The economic losses caused by neosporosis, along with the absence of effective treatment or vaccine, lead many farm owners and veterinarians to seek effective surveillance and control programs. Serologic surveillance lies at the base of any such program, and its results lead to management decisions regarding each individual cow (Dubey et al., 2007; Larson et al., 2004). The main three factors influencing the serological diagnosis of neosporosis are the methodology, the diagnostic cutoff and the timing. Individual testing of herds with hundreds of cows is a well-accepted method for evaluating in-herd seroprevalence. However, it is laborious and costly and, therefore, the timing and methodology should be carefully planned. Several serological methods have been used for the diagnosis of neosporosis, with most studies based on either IFAT or ELISA. Various studies use different diagnostic cutoff IFAT titers, ranging between 1:25 and 1:200 (Dubey et al., 2017; Dubey and Schares, 2011; Dubey et al., 2007; Rodrigues et al., 2020). In recent years, several commercial diagnostic ELISA kits have been developed, and are commonly used for routine diagnosis. Although ELISA results are calculated from continues optical density (OD) reads, they are interpreted as dichotomous diagnosis of positive or negative. This interpretation is based on the internal positive and negative controls of each kit, but the diagnostic cutoff titer is not specified. Several previous studies found varying agreement between different serological tests, which may be, partially attributed to differences in sensitivity (Baum et al., 1995; Campero et al., 2018; Reichel and Pfeiffer, 2002; von Blumroder et al., 2004; Waldner et al., 2004). In the current study, the agreement between IFAT and ELISA results was almost perfect for a diagnostic cutoff titer of 1:800, but only substantial for a cutoff titer of 1:200 (Landis and Koch, 1977). Therefore, the interpretation of the results is more reliable for a cutoff titer of 1:800 and will, most likely, detect cows with increased risk of abortion, In several previous studies from our lab, using the same IFAT protocol, only antibody titers of 1:800 or higher were significantly linked with abortions (Mazuz et al., 2014; Mazuz et al., 2015; Mazuz et al., 2021). However, cows with antibody titers of 1:200 also showed potential for vertical transmission to their offsprings (Tirosh-Levy et al., 2025). Therefore, the use of ELISA test could assist in identifying and reducing the risk of abortion and the financial consequences of infection, but is not suggested if elimination of vertical transmission or eradication of neosporosis from the herd is desired.

Exploring the dynamics of antibody titers over 18 months demonstrated fluctuations in antibody titers over time in the majority of cases, as was previously demonstrated by others (de Oliveira et al., 2023; Nogareda et al., 2007). However, most heifers maintained their initial serological status over time. These results support previous findings that congenital infection usually persists throughout the cow's life, and that the rate of horizontal transmission in endemic herds is generally low (Dijkstra et al., 2008; Hietala and Thurmond, 1999). In the current study, 67 % of the heifers that were sampled five times maintained their serological status throughout the study. These results concur with a previous report reporting 5.3 % of cows converting their ser-status from positive to negative during pregnancy (Nogareda et al., 2007). In this study, 19 of 21 heifers that seroconverted from negative to positive eventually resolved, and only three remained persistently infected in further examinations. We suppose that these cases may reflect horizontal transmission, as seropositivity was mostly transient. Nevertheless, since no avidity nor IgM testing was performed, this speculation could not be confirmed. Another possibility is that antibody levels were below the limit of detection at the time of the first testing, thus, representing undetectable persistent infection.

Since the timing of infection influences the risk of abortion (López-Gatius et al., 2004; Williams et al., 2000), the outcome of infection may be different in cases of new infection than in chronically infected dams. In addition, although *Neospora* infection increases the risk of abortions, not all individual seropositive animals show this tendency. It appears that the risk of abortions and repeated abortion is increased for some cows, but not others (Mazuz et al., 2014). Therefore, the history of each cow should be taken into consideration in the decision making following serodiagnosis.

The epidemiology of neosporosis differ between farms, with varying prevalence, vertical transmission and clinical effect. Several studies demonstrated such differences between farms (Mazuz et al., 2015, 2021; Villa et al., 2022), which may suggest that neosporosis is a multifactorial disease with clinical consequences that are influenced by environmental, immunological and husbandry factors. In this study, the prevalence and distribution of antibody titers also varied between farms. The Israeli dairy population is quite homogenous in terms of breed and genetics. Thus, these differences are probably mainly attributed to such external factors.

Choosing the timing of a serological survey is important both for reliable detection of all positive animals, which may be influenced by the fluctuating antibody titer, and for effective decision making regarding each cow. Sampling heifers prior to first insemination may allow selective breeding starting from the first pregnancy, before any potential clinical effects. Since parasite re-emergence occurs during mid-pregnancy, sampling at this stage may more accurately reflect seropositivity and the risk of abortion (Innes et al., 2001; Mazuz et al., 2014, 2015, 2021; Nogareda et al., 2007). Repeated samples may be more sensitive, and more accurately detect all positive animals, but are more costly and laborious. The results of this study suggest that sampling young heifers has good positive and negative predictive values in identifying all positive animals. In addition, this methodology will provide background information for each cow in case of future reproductive failure. This will allow discrimination between de-novo and chronic infection, which will aid the decision of removal from the herd,

Table 3
The fluctuations in anti-*Neospora* antibody titers of 91 heifers sampled at three farms on five different occasions over a period of 18 months, between the ages of 5–9 and 24–28 months, as measured by IFAT. The intensity of the color corresponds with the titer.

A. Initially seropositive

Cow	1	3	4	5	6
1069	800	200	200	800	0
1087	800	400	400	800	800
1067	800	400	800	800	200
1085	800	400	800	12,800	3,200
1347	800	800	800	800	400
1379	800	800	800	800	800
1343	800	800	800	12,800	800
1090	800	800	3,200	800	0
1065	800	800	12,800	12,800	12,800
1071	800	3,200	12,800	12,800	400
1063	800	3,200	12,800	12,800	3,200
1384	800	12,800	200	800	0
1367	800	12,800	800	400	800
1356	800	12,800	800	3,200	200
1348	3,200	0	3,200	800	3,200
1375	3,200	200	200	3,200	3,200
1358	3,200	200	800	800	200
1365	3,200	200	800	800	200
2121	3,200	400	400	800	400
1351	3,200	800	800	800	400
1089	3,200	800	800	3,200	800
1353	3,200	800	800	12,800	400
1084	3,200	800	800	12,800	3,200
2127	3,200	800	3,200	800	200
1066	3,200	800	12,800	800	400
2135	3,200	800	12,800	12,800	800
1088	3,200	800	12,800	12,800	12,800
1381	3,200	3,200	800	800	400
1370	3,200	3,200	800	800	12,800
1092	3,200	3,200	800	12,800	0
1373	3,200	3,200	800	12,800	800
1076	3,200	3,200	12,800	12,800	800
2143	3,200	12,800	800	800	800
1082	3,200	12,800	800	12,800	800
1372	3,200	12,800	800	12,800	800
2151	3,200	12,800	12,800	3,200	3,200
1079	3,200	12,800	12,800	12,800	400
1349	12,800	200	800	12,800	3,200
1070	12,800	800	12,800	12,800	800
1359	12,800	3,200	800	12,800	400
1368	12,800	3,200	800	12,800	800
1074	12,800	3,200	3,200	12,800	400
2140	12,800	3,200	12,800	800	800
1354	12,800	12,800	800	3,200	200
1105	12,800	12,800	12,800	12,800	3,200
1110	12,800	12,800	12,800	12,800	3,200
1093	12,800	12,800	12,800	12,800	12,800

B. Initially seronegative or borderline

Cow	1	3	4	5	6
1094	0	0	0	0	0
1096	0	0	0	0	0
2131	0	0	0	200	0
1060	0	0	0	200	0
2124	0	0	0	200	200
1111	0	0	200	0	0
1374	0	0	200	0	0
2149	0	0	200	400	0
1369	0	0	400	0	0
1382	0	0	400	0	0
2134	0	0	400	400	0
1371	0	0	800	0	0
2118	0	0	800	400	0
1120	0	0	800	400	0
1364	0	0	800	400	0
1115	0	0	800	400	400
1376	0	200	0	0	0
2136	0	200	400	0	0
2155	0	200	400	400	0
2156	0	200	400	800	400
1119	0	200	800	0	200
1078	0	200	800	200	0
1114	0	200	800	200	0
1344	0	200	800	200	200
2148	0	400	200	200	3,200
2153	0	400	400	0	0
2137	0	800	800	0	0
1361	0	800	3,200	800	800
1107	200	0	200	200	0
1109	200	0	400	0	0
1352	200	0	400	200	0
2158	200	200	800	0	0
1362	200	200	800	200	0
1363	200	200	800	800	0
1080	200	3,200	800	12,800	400
2119	400	200	200	0	0
1072	400	200	200	200	0
1064	400	200	400	200	0
1097	400	200	800	0	0
1366	400	200	800	800	0
1357	400	200	800	800	400
1113	400	200	3,200	12,800	3,200
1345	400	800	200	200	200
1385	400	800	200	12,800	200

as many de-novo infections may clear in the future. In addition, it may assist in identifying chronically infected cows with increased chance of aborting. However, lactating cows had considerably lower antibody titers than heifers or pregnant heifers, with decreased sensitivity and NPV of the test. Therefore, it is advisable to either sample prior to calving or wait until after the next insemination. It appears that repeated samples do not have much advantage over a single sample, when performed in the right time. These results concur with a previous study which also showed that a single sample would be satisfactory for herd screening for neosporosis (Dijkstra et al., 2003).

The total seroprevalence of *Neospora* in the farm should be taken into consideration in the construction of any control program. When the seroprevalence is low, an eradication program may be considered, including removal of seropositive cows from the herd and screening before the introduction of new animals to the herd. However, in highly endemic herds, eradication is not feasible, and the main goal is to reduce the economic impact. In such herds not all seropositive cows should be treated the same way, and the aborting cows should be individually recognized. For this reason, early diagnosis of seropositive heifers is beneficial to identify congenitally infected individuals. Future surveillance on fertility and abortions in combination with serological testing of aborting cows will allow the distinction between transient (possible new) or chronic infection and recognition of clinically affected chronically infected cows.

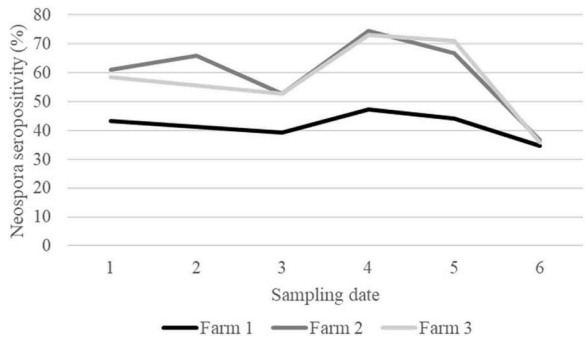

Fig. 3. Variations in *Neospora* seroprevalence in heifers sampled on three farms on six sampling dates, at three months intervals.

Table 4
The association between *Neospora* seropositivity and the sampling date, the farm (on all dates together) and the metabolic status of 132 heifers sampled between the ages of 5 and 28 months on 6 different "occasions" at three farms. Associations were assessed using Chi-square and Fisher's exact tests, and odds ratios (OR) with 95 % confidence intervals (CI) were calculated.

		N	Seropositive (%)	OR (95 % CI)	Sig
Sampling date	**1**	132	73 (55.3)	ref	ref
	2	53	35 (66.0)	1.57 (0.77–3.26)	0.192
	3	124	61 (49.2)	0.78 (0.46–1.32)	0.381
	4	126	83 (65.9)	1.56 (0.91–2.67)	0.098
	5	120	74 (61.7)	1.30 (0.76–2.22)	0.371
	6	100	36 (36.0)	0.45 (0.26–0.80)	0.003
Farm	**Farm 1**	165	70 (42.4)	ref	ref
	Farm 2	293	177 (60.4)	2.07 (1.38–3.11)	< 0.001
	Farm 3	197	115 (58.4)	1.90 (1.23–2.96)	0.003
Status	**Heifers**	366	202 (55.2)	ref	ref
	Pregnant	232	139 (59.9)	1.21 (0.86–1.72)	0.271
	Lactating	56	20 (35.7)	0.45 (0.24–0.84)	0.009

Table 5
Comparison between the serological detection of neosporosis in 91 heifers by using only a single screening **A**. at the age of 5–9 months (first screening, IFAT1) **B**. at the age of 24–28 months (last screening, IFAT6), then by using multiple screening (using the result of at least three of five tests). The sensitivity (Se), specificity (Sp), positive (PPV) and negative (NPV) predictive values of the single test were calculated in reference to repeated samples.

A		**IFAT**			**IFAT1**	
		Negative	**Positive**	**Total**	**Se**	93.8
IFAT1	**Negative**	41	3	44	**Sp**	95.3
	Positive	2	45	47	**PPV**	95.7
	Total	43	48	91	**NPV**	93.2
B		**IFAT**			**IFAT6**	
		Negative	**Positive**	**Total**	**Se**	60.4
IFAT6	**Negative**	42	19	61	**Sp**	97.7
	Positive	1	29	30	**PPV**	96.7
	Total	43	48	91	**NPV**	68.9

5. Conclusion

Construction of control programs for neosporosis rely on serological surveillance of the herd. The fluctuations in antibody titers of infected cows, may affect the results of serological surveillance. In addition, since no effective treatment is available, the timing of serological sampling could affect the decision-making regarding future breeding or management of each cow. In endemic herds, most of *Neospora* infections are congenital. Horizonal transmission occurs, but only the paucity of cases results in chronic infection. The results of the current study suggest that sampling of young heifers, after the depletion of maternal antibodies and before first insemination, has high diagnostic value. Sampling at a young age provides background information which allows future discrimination between new and chronic infection. Nevertheless, it is important to emphasize that some persistently infected heifers could have undetectable levels of antibodies at that time. In addition, herd managers may decide to either selectively breed seropositive heifers with beef cattle in order to reduce *Neospora* prevalence in future replacement heifers or to follow up on seropositive individuals to detect the ones more prone to abortions.

CRediT authorship contribution statement

Jacob Joost Doekes: Investigation. **Monica L. Mazuz:** Writing – review & editing, Investigation, Funding acquisition, Conceptualization. **Sharon Tirosh-Levy:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Elena Blinder:** Investigation.

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Declaration of Competing Interest

The authors report no conflict of interest.

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Data availability

The data presented in this study are available on request from the corresponding author.

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